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Flow Cytometric Analysis Of Hepatocytes from Normal, PFDA, and PH/DEN/PB-Treated Rats

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The goal of this project was to provide preliminary data regarding the hepatotoxic and immunotoxic effects of perfluorodecanoic acid (PFDA). Flow cytometric evaluation of hepatocytes from PFDA-treated rats revealed an increase in size and granularity and a shift from tetraploidy towards diploidy, in rats treated with 50 mg/kg PFDA, as well as a suggestion of interference with lymphocyte blast transformation of splenocytes in these rats. These preliminary studies suggest that PFDA induces hepatocellular alterations which are detectable by flow cytometry, and induces both morphologic and possibly functional modulation of the immune system. Further studies are indicated to determine the significance of these findings.

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I. Introduction

This project was designed to generate preliminary information regarding the toxic and potential carcinogenic effects of perfluorodecanoic acid (PFDA) on rat hepatocytes. These studies were performed in collaboration with some pharmacokinetic studies (using the same animals) being performed by personnel of the Toxic Hazards Division, AAMRL, Wright Patterson AFB. As more experiments were done with PFDA, new information was generated which caused us to deviate from the original objectives (listed below). There were also logistical problems of transporting rat tissues from WPAFB to our laboratory as well as a shift of emphasis at WPAFB from PFDA to other compounds, so the amount of work we were able to perform was far less than we had hoped. Nonetheless, some interesting and promising data was generated which would certainly warrant further studies.

II. Objectives

The objectives as presented in the original proposal are presented below:

A. Develop or purchase monoclonal or polyclonal antibodies to various hepatocellular enzyme markers which reportedly appear (or disappear) during carcinogenesis.

B. Using flow cytometry, evaluate hepatocytes from normal rats, PFDA-treated and pair-fed control rats, and rats treated with a known initiator (diethylnitrosamine (DEN)) and promoter (phenobarbital (PB)) at various times

following treatments. Parameters to be evaluated were enzyme expression, nuclear ploidy (DNA content) and cell cycle.

C. Develop monoclonal antibodies against hepatocytes from PFDA-treated rats for the purpose of detecting the appearance or loss of antigens on treated rat hepatocytes compared to normal rats.

Because some of the original objectives could not be met (see below) alternate objectives were added which include:

D. Evaluate changes in morphology of lymphoid organs (thymus, spleen) in PFDA treated rats.

E. Evaluate the lymphocyte blast transformation (LBT) response of splenocyte from PFDA-treated rats.

III. <u>Status of Research</u>

A. Development of monoclonal antibodies (MoAb) against hepatocellular enzymes:

1. Rationale: In the well characterized two-stage model of hepatocarcinogenesis, exposure to an initiator chemical (such as DEN) and then a promoter (such as PB) results in preneoplastic alterations in hepatocellular enzyme expression. Our intention was to develop antibodies against these known enzyme markers so that we could use flow cytometric analysis to compare early enzyme alterations of hepatocytes from DEN/PB treated rats to those from PFDA-treated rats.

2. Results: Work at WPAFB as well as published reports

from other laboratories working with similar compounds yielded mounting evidence that PFDA does not induce hepatocellular enzyme changes similar to those induced by DEN/PB. It was decided not to go to the expense of generating MoAb until it could be established which, if any, hepatocellular enzymes are altered with PFDA treatment. Dr. Frank Witzmann, Indiana-Purdue University, has been working in collaboration with Miss Marilyn George at WPAFB to identify and isolate PFDA-induced hepatocyte enzyme or protein changes, but this work objective had not been accomplished before our grant expired.

B. Flow cytometric evaluation of hepatocytes from PFDA treated rats:

1. Rationale: There are several morphologic and biochemical changes known to occur in cells during preneoplastic events. For parameters which can be measured on cell suspensions, flow cytometry provides rapid and accurate evaluation of up to six parameters per cell and of the percent and degree of alterations in the entire cell population. The parameters which we actually measured for hepatocytes from PFDA-treated rats were hepatocyte size, granularity, and DNA content.

2. Methods:

a. Animal Treatment: Male Fischer 344 rats were

injected once (day 0) intraperitoneally (i.p.) with 20mg/kg or 50mg/kg PFDA. These rats were fed ad lib until day 8 or 30, whereupon their livers were perfused and hepatocytes harvested. Pair-fed control rats were injected with vehicle control (propylene glycol / H_2O, 1:1) and given the same amount of food consumed by PFDA treated-rats and their livers perfused and hepatocytes harvested after 8 or 30 days. Normal rats received food ad lib from entire time periods before sacrifice.

8 day Expt.	30 day Expt.
June 1988	Sept 1988
Jan. 1989	
April 1989	
July 1989	

b. Hepatocyte Isolation: Hepatocytes from PFDA-treated, pair-fed control, and normal rats were isolated by a heparin and collagenase perfusion technique as described by Seglen[1] and Oldham et al.[2] and modified by DelRaso[3]. Briefly, male Fischer 344 rats were anesthetized by intraperitoneal injection with a mixture of ketamine and xylazine. The rat abdomen is opened asceptically and the posterior vena cava located and cannulated with a 22 gauge indwelling catheter. The liver was perfused for approximately 10 minutes with heparin supplemented

media and then approximately 15 minutes with a collagenase supplemented media. The liver was removed and single cells expelled by making incisions into the liver and teasing with a comb. Stromal tissue was removed by successive filtrations through both 250 and 60 micron pore size monofilament nylon mesh. Cells were then subjected to percoll gradient fractionation to isolate parenchymal cells. This resulted in two hepatocellular fractions (top and bottom) from PFDA-treated rats. Cells were washed twice, resuspended in L-15 media, and their viability determined by trypan blue exclusion. These cells were placed on ice and delivered to the Ohio State University Dept. of Veterinary Pathobiology.

c. Hepatocyte Nuclei Isolation: Hepatocyte nuclei were isolated from hepatocytes by suspending pelleted cells in 0.1% (v/v) Nonidet P-40 (NP-40) detergent diluted in phosphate buffered saline (PBS) solution and incubated on ice for 15 minutes. Two percent paraformaldehyde was added to the above nuclei/cell suspension and slowly mixed for 15 minutes at 25°C. Hepatocyte nuclei were then pelleted, resuspended in PBS, and passed through 50μm nylon mesh.

d. Hepatocyte Nuclei Staining: Nuclei were stained with a 50μg/ml propidium iodide and RNase (100U/ml)

in NP-40/PBS buffer for 15 minutes and evaluated by flow cytometry.

- Flow Cytometric Evaluation: Whole hepatocytes were analyzed by an EPICS 753 flow cytometer measuring both forward angle light scatter (FALS:cell size) and 90° light scatter (LI90:cell granularity). Nuclei were evaluated using FALS, LI90, integrated red fluorescence (IRFL) and peak red fluorescence (PRFL). Ten thousand events were collected and stored as list nodes on the MDADS unit (EPICS) for subsequent evaluation.

3. Results: The hepatocellular changes induced by exposure to PFDA as evaluated by flow cytometry are presented in Tables I and II. At 50mg/kg PFDA increased both the size (Table I) and granularity (Table II) compared to pair-fed controls. These hepatocytes also exhibited an alteration in their DNA content compared to both normal and pair-fed control rats as measured by propidium iodide staining (Table III). This alteration in DNA content was observed by an increase in the diploid population distinct from the normal tetraploid population that exists in the rat liver.

4. Significance: The changes in hepatocellular size and granularity evaluated by flow cytometry correlate with those observed by Van Rafelghen et al.[4] using light and electron microscopy. This

group found that peroxisome proliferation in PFDA-treated rats was approximately 50 times normal. We also observed the induction of a diploid hepatocellular population similar to that reported by several investigators[5,6] using known hepatic carcinogens. Whether PFDA, a peroxisome proliferator, induces hepatocarcinogenesis in rats has not been fully determined. However, the changes observed in PFDA-treated rat hepatocytes may reflect the carcinogenic potential of this compound.

C. Development of monoclonal antibodies against hepatocytes from PFDA-treated and normal rats.

1. Rationale: The goal of this experiment was to raise antibodies against hepatocytes from PFDA-treated and normal rats, then absorb both antibodies with normal rat hepatocytes. The absorbed antibodies would then be incubated with hepatocytes from normal and PFDA-treated rats, and evaluated by flow cytometry to detect any differences in antigen expression between the two cell populations. This was intended as an adjunct to the enzyme marker experiments.

2. Results: This objective was not accomplished or even attempted for several reasons. The main reason is that Dr. Witzmann began doing similar experiments (mentioned above) but by different techniques. In addition, the paucity of tissues from treated rats, and a high probability of failure for this

particular experiment, were further reasons to delete this objective.

D. Evaluation of lymphoid organs in PFDA-treated rats:

1. Rationale: Our interest in immunotoxicology motivated us to do these experiments since the tissues were readily available and would have been discarded. PFDA has similar general toxic effects as does tetrachlorodibenzo-p-dioxin (TCDD), which has striking immunotoxic effects; thus we felt that preliminary immunotoxicity studies for PFDA were justified.

2. Methods: Spleen and thymus were collected from five groups of three rats each: 50mg/kg PFDA, 20mg/kg PFDA, the respective pair-fed controls, and normal rats. All rats were sacrificed 8 days after a single PFDA oral administration. The spleens and thymuses were fixed in 10% buffered formalin, then sectioned and stained with hematoxalin and eosin for microscopic evaluation.

3. Results: All rats in the 50mg/kg PFDA dose group showed moderate to marked lymphoid depletion of the thymus (thymic atrophy) (Table IV). The spleen of this group, and the spleen and thymus of all other treatment or pair-fed groups, did not differ significantly from those of the normal rats.

4. Significance: Since the thymus is in essence ultimately responsible for cell-mediated immunity,

marked thymic atrophy as was seen in the 50mg/kg PFDA dose group would be associated with and/or result in profound suppression of cell mediated immune functions. Thymic atrophy also occurs as a result of TCDD exposure, which is known to induce a primary deficit in cell-mediated immunity. It would be of interest to evaluate all lymphoid tissues after longer exposure times to PFDA, as well as a wider dose range.

E. Effects of PFDA on the lymphocytes blast transformation (LBT) response.

1. Rationale: The LBT assay is a simple and widely used assay which evaluates the ability of T and B lymphocytes to proliferate in response to mitogen or antigen stimulation. Since the spleens were available from the PFDA-treated rats, we evaluated the splenic LBT response to the mitogens concanavalin A (Con A) and lipopolysaccharide (LPS).

2. Methods: Spleens were harvested from PFDA-treated or pair-fed controls, or normal rats after liver perfusion. A single cell suspension was prepared by passing tissue through a #60 wire mesh screen and aspirating two successive times through each 18, 23 and 27 gauge needles. Red cells were lysed using an 0.15 ammonium chloride and cells were washed two times and resuspended in RRMI 1640 (supplemented with 5% fetal bovine serum, 2% $NaHCO_3$, 1% penicillin

and streptomycin with mycostatin $1x10^6$ cells/ml. Cells were plated in 100ml ($1x10^5$ cells/well) quantity into a 96 well microculture plate. Cell culture medium alone or with Con A (5μg/ml) or LPS (20μg/well) was added to cells in quadruplicate. Cells were incubated at 37°C in 5% CO_2 humidified incubator for 72 hours. The lymphoproliferative response was determined by 6 hour incorporation of [^{3}H]-thymidine prior to harvest and then counted by liquid scintillation spectrometry.

3. Results: Table V gives the results of two separate lymphoblastogenesis experiments. The results of the two experiments were quite different in their outcome and thus are reported separately. In experiment #1 PFDA caused a significant decrease in response to the T cell mitogen (Con A; at 20mg/kg) and to the B cell mitogen (LPS; at both 20 and 50 mg/kg) over control. However, in experiment #2 PFDA significantly enhanced Con A induced blastogenesis in 20 and 50 mg/kg PFDA treated rats and increased LPS-induced blastogenesis of 20 mg/kg PFDA.

4. Significance: Due to the variable effect reported in both of the experiments represented in Table V, the importance of in vivo exposure PFDA on lymphocyte blastogenesis is unclear. PFDA exposure does induce immunomodulation, but further experimentation is needed to determine the

significance of exposure to this chemical on the immune system.

Table I. Flow Cytometric Analysis of Hepatocytes Isolated from PFDA-treated or Pair-fed Control Fischer 344 Rats.

Forward Angle Light Scatter (FALS)
Relative Size

	8 day		30 day
	mean[a]	s.d.	mean[b]
20 mg/kg Pair-fed (Control)	38.6[c]	11.4	37.6
20 mg/kg PFDA (Top)	45.8[c]	6.7	48.9
20 mg/kg PFDA (Bottom)	42.1[c]	5.9	49.1
50 mg/kg Pair-fed (Control)	31.0[d]	4.0	34.6
50 mg/kg PFDA (Top)	42.6[d,e]	5.6	50.7
50 mg/kg PFDA (Bottom)	25.7[d]	9.4	42.1

[a] n = 4, except at 50mg/kg PFDA (Bottom) n=2
[b] n = 1
[c] P = .509, means are not significantly different (ANOVA)
[d] P = .220, means are significantly different (ANOVA)
[e] P = .015, mean is significantly different from control (student's T-test)

Table II. Flow cytometric Analysis of Hepatocytes Isolated from PFDA-treated or Pair-fed Control Fischer 344 Rats.

Log Integrated 90° Light Scatter (LI90)
Relative Cellular Granularity

	8 Day Treatment		30 Day Treatment
	mean[a]	s.d.	mean[b]
20 mg/kg Pair-fed (Control)	43.9[c]	10.5	66.6
20 mg/kg PFDA (Top)	57.9[c]	6.4	72.9
20 mg/kg PFDA (Bottom)	55.3[c]	7.6	71.7
50 mg/kg Pair-fed (Control)	39.9[d]	6.3	54.1
50 mg/kg PFDA (Top)	58.9[d,e]	8.2	72.1
50 mg/kg PFDA (Bottom)	44.5[d]	27.4	66.5

[a] n = 4, except at 50 mg/kg PFDA (Bottom) n = 2
[b] n = 1
[c] P = .091, means are not significantly different (ANOVA).
[d] P = .154, means are not significantly different (ANOVA)
[e] P = .010, mean is significantly different from control (student's T test)

Table III. Ploidy Distributions in Hepatocyte Nuclei from Normal, Pair-fed, and PFDA-treated Rats.

	% Hepatocytes with DNA Content		
	2N	4N	8N
Normal	4.2	62.3	17.1
Pair-fed			
(20)	7.3	59.9	9.7
(50)	3.4	61.0	16.9
PFDA-treated			
20 mg/kg (top)	5.1	55.1	12.5
20 mg/kg (bottom)	6.6	63.0	12.1
50 mg/kg	3.6	43.1	11.4

Table IV. Effects of PFDA on Histology of Spleen and Thymus[a]

	Normal	20mg/kg PFDA	20 pair-fed	50mg/kg PFDA	50 pair-fed
Expt.1					
thymus	NSML[b]	NSML	NSML	marked depletion	NSML
spleen	NSML	NA[c]	NSML	NA	NSML
Expt. 2					
thymus	NSML	NSML	NSML	moderate depletion	NSML
spleen	NSML	NSML	NSML	NSML	MSML
Expt. 3					
thymus	NSML	NSML	NSML	marked depletion	NSML
spleen	NSML	NSML	NSML	NSML	NSML

[a]Rats were sacrificed 8 days after PFDA treatment or pair feeding.
[b]NSML = No significant microscopic lesions
[c]NA = Not available for evaluation

Table V. Rat Lymphocyte Blastogenesis in PFDA-treated and Pair-fed Control Fischer 344 Rats.

a. Experiment #1 (April 1989)

	3H-Thymidine Uptake		
	Control	Con A	LPS
Normal	650 ± 156	41225 ± 8276[a,c]	11937 ± 695[b,d]
Pair-fed (20)	748 ± 167	50012 ± 1555[a]	12433 ± 2061[b]
PFDA (20mg/kg)	376 ± 125	26793 ± 3553[a]	7423 ± 1229[b]
Pair-fed (50)	431 ± 60	48780 ± 4490[c]	9008 ± 1391[d]
PFDA (50mg/kg)	431 ± 54	33834 ± 8248[c]	6137 ± 1684[d]

b. Experiment #2 (July 1989)

Normal	353 ± 75	92846 ± 11538[a,c]	4519 ± 712[b,d]
Pair-fed (20)	202 ± 69	99876 ± 3483[a]	3265 ± 756[b]
PFDA (20mg/kg)	384 ± 65	123293 ± 8169[a]	11862 ± 1580[b]
Pair-fed (50)	189 ± 52	147674 ± 6208[e]	2733 ± 424[d]
PFDA (50mg/kg)	188 ± 20	151289 ± 16039[e]	5348 ± 788[d]

[a]P = .0016 means are significantly different (ANOVA)
[b]P < .001 means are significantly different (ANOVA)
[c]P = .110 means are not significantly different (ANOVA)
[d]P < .0616 means are significantly different (ANOVA)
[e]P = .0001 means are significantly different (ANOVA)

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VI. Oral Presentations

A. Frazier, D.E., Tarr, M.J., Huff, L.Y., George, M.E., and Anderson, M.E.: Hepatocellular Changes Induced by Perfluorodecanoic Acid (PFDA). Presented at Seventh Annual Epics Users Meeting, Arlington, Virginia, May 2-4, 1989.